Signal Amplification Methods for Analyte Detection

CROSS-REFERENCE TO RELATED APPLICATION(S)

[001] This application claims priority to U.S. Provisional Application Nos. 60/532,088 and 60/532,089 filed December 23, 2003, and 60/540,576, filed on January 31, 2004. The entire disclosure of the prior applications is considered to be part of the disclosure of the instant application and is hereby incorporated by reference.

STATEMENT REGARDING RESEARCH & DEVELOPMENT

TECHNICAL FIELD

[002] This methods and compositions provided herein relates to improvement of methods for analyte detection using a signal amplification system (SAS). The SAS is composed of a structure consisting of (a) multiple signaling moieties such as chemiluminescent compounds, (b) a carrier entity or entities and (c) one or more analyte binding moieties.

BACKGROUND OF THE INVENTION

[003] Detecting biological analytes such as bacteria, antigens, antibodies, receptors, ligands and nucleic acids is pivotal to diagnostic test methods for a wide variety of diseases and conditions and is important to research, forensic and risk assessment applications. Such methods typically rely on specific binding between a target biological analyte and a corresponding analyte binding molecule to form a complex that can be readily detected. For example, bacteria may be detected by binding to particular lecithins or antibodies specific for surface antigens on the bacteria. Soluble antigens may be detected by binding to specific antibodies raised against the antigen. Conversely, specific antibodies may be detected by binding to their corresponding antigens (or antigen conjugates). Receptors on cell surfaces indicative of particular cell types may be detected by binding to their corresponding

ligands. Conversely, ligands may be detected by binding to their corresponding receptors. Nucleic acids may be detected by hybridizing to substantially complementary nucleic acid sequences. Central to all these detection methods is the ability to detect the formation of a bound complex between the target analyte and the analyte binding molecule, which is distinguishable from non-complexed molecules. Typically the bound complex is detected by one of three basic techniques.

[004] The first basic technique relies on a change in the physical state of the analyte binding molecule complexed with the analyte relative to either component alone. A common example of this technique is antibody agglutination, whereby an antibody or an antibody bound to a particle, such as a latex particle, becomes interconnected through cross binding to form a lattice of sufficient size to scatter light in comparison to non-analyte bound antibodies. A common form of an agglutinating assay relies on adhering an antibody to the surface of a microtiter plate and contacting the same with a sample containing the analyte and a reagent having a second antibody linked to latex particles. After a sufficient period of incubation, a light scattering lattice comprised of the latex particles complexed with the analyte forms on the surface of the microtiter plate, and the size of the lattice is measured by light scattering or absorption. One disadvantage of agglutinating assays and similar physical state based methods is that they are relatively insensitive, requiring a relatively large amount of analyte material to reliably detect the complex.

[005] The second basic technique for detecting an analyte complex is the ELISA method, which relies on linking an enzyme to an antibody. The enzyme linked species forms a sandwich complex with the analyte and another antibody (or antigen) species typically immobilized on a surface. After washing the surface bound complex to remove unbound enzyme-linked molecules, the bound complex is incubated with a substrate for the enzyme to detect the conversion of the substrate to a product that is measured by conventional spectrophotometeric or chemoluminescent techniques. ELISA methods provide the benefit of relatively high sensitivity, but have the disadvantage of taking a relatively long time to execute to obtain maximum sensitivity. In a typical ELISA test, the antibody-antigen

binding may require several hours (typically overnight) to reach equilibrium (100% completion) while a 10-minute incubation might reach only about 3% completion. ELISA test are therefore not practical for rapid on site testing in a short time frame, such as for rapid screening patients or blood donors for diseases. ELISA tests also have other disadvantages such as instability of the linked enzyme, relatively expensive substrates and requiring multiple steps to execute, all of which lead to relatively high costs for ELISA tests.

[006] The third basic analyte complex detection technique is labeling, which relies on detecting a label attached to the analyte binding molecule after it is bound to the analyte. Typically the analyte sample is immobilized on a substrate, incubated with the labeled analyte binding molecule, and then washed to remove unbound labeled molecules. Labeling techniques are most commonly used with nucleic acid detection methods where the analyte binding molecule is a nucleic acid probe that is hybridized to a complementary sequence of the target analyte nucleic acid. A variety of label types have been used in this regard, including for example, radioactive, fluorescent, chemiluminescent and electroluminescent species. A variety of substrates have also been used, from simple filter-like membranes to complex nucleic acid chip arrays. In the clinical arena, the most commonly used nucleic acid binding tests are for screening blood for viruses (*e.g.*, HIV, HCV and HBV) or for HIV viral load testing. Viral load tests are used to measure viral concentration in the plasma as a means to monitor effectiveness of anti-viral drug therapy or disease progression. Nucleic acid labeling techniques are frequently used in conjunction with electrophoresis or other size separating procedures to identify target analyte molecules of particular sizes.

[007] One of the major disadvantages of conventional labeling techniques, especially those associated with detecting nucleic acids is that they are relatively complex, requiring skill and training to execute. Another disadvantage is that like ELISA tests, labeling test requires a relatively long incubation period to drive the hybridization to a sufficient level to detect the label. Another related disadvantage is that the amount of labeled signal molecules attached to the probe is limited by the

size of the probe and the necessity of protecting the hydrogen bonding domains for hybridization. This limits the sensitively of detection, which is sometimes addressed by analyte amplification techniques such as PCR (polymerase chain reaction) to amplify the target analyte nucleic acid. PCR adds another level of complexity (and variability) associated with the enzymes, reagents and protocols needed for reliable PCR. Yet another disadvantage is that relatively expensive equipment is required for the most sensitive types of detection. These disadvantages effectively proscribe the use of conventional labeling techniques from applications requiring rapid and inexpensive on-site detection at the time of service, such as required in the clinical arena.

[008] Accordingly, there is a need in the art for compositions and methods for improving the sensitivity, speed and simplicity of analyte detection, and especially for such compositions and methods that are readily adaptable for detecting a wide variety of analytes.

SUMMARY OF THE INVENTION

[009] In one aspect, there are provided compositions for analyzing a sample for the presence of an analyte. In various embodiments, there are provided, a carrier for detecting an analyte in a sample, that includes an analyte binding moiety and a signaling moiety each associated with the carrier, where the analyte binding moiety is associated with the carrier so that it is disposed away from the carrier a greater distance than the signaling moiety is disposed from the carrier.

[010] In typical embodiments, the carrier is a particle, and the signaling moiety is encapsulated in the particle and the analyte binding moiety is associated with a surface of the particle. In other embodiments, the carrier is a particle, and the signaling moiety is associated with the particle through a first linker and the analyte binding moiety is associated with the particle through a second linker that has a longer dimension than the first linker. The analyte binding moiety and the signaling moiety can be associated with the carrier through a common linker where the signaling moiety is associated with a side group of the linker and the analyte binding moiety is associated with an end portion of the linker. The signaling moiety can be a

chemiluminescent moiety, an electrochemiluminescent moiety, a fluorescent moiety, a chromogenic moiety or an enzyme. In certain embodiments the signalling moiety comprises a rare earth element or acridinium. In other embodiments, the signaling moiety comprises europium. In certain particular embodiments, the carrier is a particle and the acridinium is encapsulated in the particle.

[011] In other embodiments, there is provided a carrier for detecting an analyte in a sample that includes an analyte binding moiety associated with the carrier; and a signaling moiety that is releasably associated with the carrier when the carrier is treated to a releasing condition. In certain embodiments, the signaling moiety is releasably associated with the carrier through a linker. The linker may be a dissociable linker. In one embodiment, the dissociable linker comprises a first nucleic acid sequence that hybridizes to a second nucleic acid sequence attached to the carrier. In other embodiments, the linker is a cleavable linker. The cleavable linker may contain a photolabile linkage, an enzymatically cleavable linkage, or a chemically cleavable linkage. In certain embodiment, the cleavable linker comprises at least one of a disulfide linkage.

[012] In various embodiments, the carrier is a particle, and the signaling moiety is encapsulated in the particle and the releasing condition is physical crushing of the particle. In other embodiments, the releasing condition comprises at least one of swelling or solubilization of the particle.

[013] In particular embodiments, the carrier is a particle, and the analyte binding moiety is associated with a surface of the particle and the signaling moiety is encapsulated within the particle. In particular embodiments, the signaling moiety is acridinium. The signaling moiety in various embodiments can be any of the types of signalling molecules mentioned above.

[014] A various other embodiments there is provided a carrier for detecting an analyte in a sample, comprising an analyte binding moiety and a signaling moiety linked to one another through a first linkage and linked to the carrier though a second linkage different from the first linkage. In some embodiments, the signaling moiety is linked to the carrier through the second linkage. In other embodiments, the analyte binding moiety is linked to the carrier through the second linkage. In some embodiments first linkage is though a linking molecule that extends the

analyte binding moiety away from the signaling moiety. In certain embodiments, the second linkage is though a linking molecule that extends the analyte binding moiety and signaling moiety away from the carrier.

[015] In any of the foregoing embodiments, when the signaling moiety comprises a rare earth element the rare earth element can be associated with the carrier with a chelating moiety. In some embodiments, at least one of the first and second linkages comprise a linker containing the chelating moiety that binds the rare earth element.

[016] In another embodiment, there is provided carrier for analyzing a sample, comprising a microparticle having acridinium encapsulated therein.

[017] In another aspect, there are provided methods for analyzing a sample for the presence of an analyte. In various embodiments, the methods comprise contacting the sample with a first analyte binding moiety associated with a substrate to form a bound complex on the substrate; contacting the bound complex with a carrier comprising a second analyte binding moiety and a signaling moiety that is releasably associated with the carrier when the carrier is treated to a releasing condition; removing carriers that do not bind the analyte and retaining carriers that do bind the analyte on the substrate; releasing the signaling moiety from the retained carriers; and detecting the released signaling moiety.

[018] In typical embodiments, the substrate is a particle. The particle can be a magnetic particle. In various embodiments, the signalling moiety can be releasably associated with the carrier through a linker. The linker can be a dissociable linker. The dissociable linker may comprises a first nucleic acid sequence that hybridizes to a second nucleic acid sequence attached to the carrier. In other embodiments, the linker is a cleavable linker. The linker can be any of the types of cleavable linkers mentioned above. In certain embodiments, the carrier is a particle, and the signaling moiety is encapsulated in the particle and the releasing condition is physical crushing of the particle. In other embodiments the releasing condition comprises at least one of swelling or solubilization of the particle. In some embodiments, the carrier is a particle, and the analyte binding moiety is associated with a surface of the particle and the signaling moiety is encapsulated within the particle.

[019] In any of the methods provided herein, multiple first carriers may be further linked to a second carrier. The methods provided herein can use any of the types of

signaling moieties mentioned above, and all result in amplifying of the detectable signal.

[020] In certain embodiments of the methods, the carrier further comprises a first binding partner that binds to a second binding partner other than the analyte. In these embodiments, the methods further includes contacting the retained carrier with a second carrier associated with a second binding partner that binds the first binding partner and with the signaling moiety that is releasably associated with the second carrier to form a multi-carrier complex. Prior to releasing the signal moieties, the second carriers not in the multi carrier complex are removed and the signal moieties in the retained carriers are released and detected.

[021] For further amplification, the second carrier may further include a third binding partner that binds to a fourth binding partner different from first and second binding partners. In these embodiments the methods further include, contacting the second carrier with a third carrier comprising the fourth binding partner and the signaling moiety releasably associated with the third carrier to form a second multi carrier complex; prior to releasing the signal moieties, removing the third carriers that are not in the second multi carrier complex and retaining the second multi carrier complex; and releasing the signal moieties from the second multi carrier complex for detection.

[022] In a different group of embodiments of a method for analyzing a sample for the presence of an analyte, the methods include contacting the sample with a first carrier associated with an analyte binding molecule and a releasable adapter having a first domain comprising a first binding partner that binds a second binding partner, and a second domain comprising a third binding partner that binds a fourth binding partner other than the second binding partner; removing the first carriers that are not bound to the analyte and retaining the first carriers that are bound to the analyte; releasing the releasable adapter from the retained first particles; contacting the released adapter with a second carrier associated with a releasable signaling moiety and with the second binding partner that binds the first binding partner of the released adapter; contacting the second carrier with a substrate that is linked to the fourth binding partner that binds the third binding partner of the released adapter to form a substrate carrier complex; removing the second carriers that are not

associated with the substrate carrier complex; releasing the signal moieties from the second carriers; and detecting the released signal moieties.

[023] In various embodiments, the second carrier is a particle, and the signaling moiety is encapsulated in the particle and the releasing condition can be any of those mentioned above. Again, in certain embodiments, the signaling moiety is acridinium.

[024] In yet other embodiments there are provided, methods for analyzing a sample for the presence of an analyte, that includes contacting the sample with a first carrier associated with an analyte binding molecule releasably associated with the carrier, and where the analyte binding molecule has a first domain that binds the analyte and second domain that comprises a first binding partner that binds a second binding partner; contacting the first carrier with a substrate that binds the first carrier; removing the first carriers that are not bound to the substrate and retaining the first carriers that are bound to the substrate; releasing the releasable analyte binding moiety from the retained first carriers; contacting the released analyte binding moiety with a second carrier associated with a releasable signaling moiety and containing a second binding partner that binds the first binding partner on the released analyte binding molecule; contacting the second carrier with a substrate that is associated with a third binding partner that binds a fourth binding partner on the analyte binding molecule; removing the second carriers that are not associated with the substrate and retaining second carriers that are associated with the substrate; releasing the signal moieties from the retained second carriers; and detecting the released signal moieties. Again these methods provide for greatly enhanced signal amplification. In typical embodiments, the carrier is a particle, and the signaling moiety is encapsulated in the particle and the releasing condition comprises at least one of swelling or solubilization of the particle. In typical embodiments, the encapsulated signaling moiety is acridinium. Typically, the second carrier is a particle, and the analyte binding moiety is associated with a surface of the particle and the signaling moiety is encapsulated within the particle.

[025] More generally, the compositions, and methods provided herein are used to increase the sensitivity of analyte detection assays that are based on using analyte binding moieties, and signal moieties attached to carriers. In order to increase the

sensitivity, it is desirable to increase the number of signal moieties detected for each bound analyte molecule. For example, a method with a 10:1 molar ratio of signaling moieties to bound analyte will be more sensitive than one with 1:1 ratio. With the current compositions and methods, a carrier is used to increase this molar ratio. A carrier can be a microparticle such as microsphere, which contains multiple sites to which analyte binding moieties (e.g., ligands) and multiple signaling moieties such as fluorescent molecules can be attached. Alternatively, a great number of fluorescent molecules can be encapsulated within microspheres whereas the analyte binding moieties (ligands) can be attached to the surface of the particles. When an analyte binding moiety binds to an analyte molecule, multiple signaling moieties on the carrier are associated with each bound analyte thereby increasing the sensitivity. An analyte-analyte binding moiety-/ carrier / signaling moiety complex can then be captured on to a solid surface to which a second analyte binding moiety is attached in an otherwise typical sandwich assay. The higher the ratio of signaling moieties to carrier, the greater the signal is normally amplified.

[026] The present disclosure also generally addresses problems the present inventors have discovered that occur when a carrier contains too many signaling moieties in close proximity. For example, fluorescent compounds tend to quench one another when they are confined in a close proximity resulting in decreasing of fluorescent signal. When encapsulated in microparticles or the like, fluorescent signal molecules may also quench one another if there are too many signal molecules in a microparticle. For those signaling moieties that require no excitation light (e.g., chemiluminescent compounds, electrochemoluminecescent compound and enzymes), problems may still exist in that they have to be in direct contact with trigger conditions such as reagents for chemoluminescent compounds or electricity for electrochemoluminecescent compounds.

[027] The applicants now provide methods and compositions that solve these problems. On of the key aspects of the current methods and compositions is to release the signaling moieties from the carriers before detection. By freeing the signaling moieties from the carriers, one solves the problems of quenching or limited accessibility to trigger reagents, or both, and substantially increase the number of detectable signaling moieties that are specifically captured during an assay.

[028] Another key aspect of the methods and compositions provided herein is use of multiple rounds of amplification in a system involving one or more carriers, each linked to multiple signalling moieties. This aspect allows even higher level of amplification of signal. Yet another key aspect of the methods and compositions is encapsulation of chemiluminescent compounds in microparticles and its use in analyte detection.

[029] Still another key aspect are methods for encapsulating acridinium or its derivatives into microparticles or other carriers. Encapsulation of acridinium or its derivatives into microparticles greatly enhances signal amplification while simplifying manufacturing processes.

[030] In general, signaling moieties are coupled to or encapsulated in a carrier, which is coated or coupled with analyte binding moieties, and/or that includes an adaptor that permits specific binding of a second carrier containing multiple signaling moieties. Detection of an analyte involves the binding of an analyte to a capture solid surface substrate (e.g., magnetic particles, microtiter plates and the like) that is coated with an analyte specific ligand (analyte binding moiety). Unwanted substances and unbound analytes are then removed through washing or similar procedure. A complex comprising signaling moieties, carrier and analyte binding moieties, is then placed in contact with solid surface with the captured analytes. The complex is immobilized on the solid surface through analyte binding. After removal of unassociated carriers through washing, the bound signaling moieties are released from the carrier and then detected with desired means such as with a fluorometer for fluorescent moieties, a luminometer for chemiluminescent moieties, a spectrophotometer for chromogenic moieties, or other suitable instrument for the type of signaling moiety used.

[031] A wide range of other well known methods can be used to separate unbound components from the complex bound with the analyte. These methods include, but are not limited to, chromatography and its variations, microsphere techniques, magnetic particle techniques, flow cytometry methods, lateral flow techniques, solid phase immobilization, solid phase extraction, centrifugation, electrophoresis and filtration. The signaling moieties can be subsequently released from the carrier for detection using physical or chemical means .

BRIEF DESCRIPTION OF THE DRAWINGS

[032] FIG. 1 is a schematic drawing of one specific embodiment of the methods and compositions provided herein, where the signaling moiety 1, e.g., chemiluminescent compound, and the analyte binding moiety 3 are attached to the carrier 2.

[033] FIG. 2 is a schematic drawing of one specific embodiment of the methods and compositions provided herein, where the analyte binding moiety 3 is attached to the signaling moiety 1.

[034] FIG. 3 is a schematic drawing of another specific embodiment of the methods and compositions provided herein, where signaling moiety 1 is attached to the analyte binding moiety 3.

[035] FIG. 4 is a schematic drawing of a preferred embodiment of the methods and compositions provided herein, where the signaling moiety 1, or their derivatives, is encapsulated in microparticles.

[036] FIG. 5 depicts another preferred embodiment of the methods and compositions provided herein, where the analyte binding moiety 3 or signaling moiety 1 or both are coupled to an intermediate carrier 4 (the second carrier), which is subsequently coupled to the first carrier 2.

[037] FIG. 6 is a schematic drawing of a layered or directional SAS, in which the signaling moiety 1 and analyte binding moiety 3 are directionally labeled on the carrier 2 via a polymer backbone 5.

[038] Fig 7 depicts an example where one end of a linear polymer molecule 5 is attached to a microsphere 2 while the other end is attached with an analyte binding moiety (*i.e.*, biotin). Signaling moieties 1 are conjugated to the functional groups in between the two termini of the polymer. In this specific example, biotin in the analyte binding moiety can be used to link antibodies (AB) conjugated with avidin or streptavidin.

[039] Fig 8 depicts an example of analyte detection using the SAS, where a microwell plate is used as the solid phase support.

[040] Fig 9 depicts another example of analyte detection using the SAS, where the magnetic particles are used as solid phase support.

[041] Fig 10 illustrates an example of a two round amplification process, where one magnetic capture particle type is used.

[042] Fig 11 illustrates another example of two round amplification process, where two magnetic capture particle types are used.

[043] Fig 12 illustrates yet another example of a two round amplification process, where the analyte binding moiety itself is used as SAS releasable linker.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

[044] Prior to describing various exemplary embodiments in detail, to aid one of ordinary skill in the art in understanding the methods and compositions provided herein, the following terms are defined herein as a reference. The definitions are provided for convenience only, and do not limit the ordinary meaning of the terms as would be understood by one of ordinary skill in the art, unless the definitions provided below conflict with such ordinary meaning, in which case the definition provided herein control.

[045] The terms "analyte binding molecule" and "analyte binding moiety" are used interchangeably to mean a molecular species having a domain that binds to a desired analyte. Example analyte binding moieties include, but are not limited to, nucleic acids, antigens, antibodies, ligands, lecithins, receptors and the like.

[046] The term "associated with" means a molecular species is associated in a physical complex with a carrier and will remain in the complex without specifically treating the complex to conditions intended to release the molecular species. The complex may be through covalent or non-covalent bonds between the species, or through physical encapsulation of one species within a matrix of the other.

[047] A "binding partner" is one member of a binding complex between at least two different molecular species. Common examples of binding partners, are antibody / antigen, mouse-anti-rabbit antibody / rabbit antibody, an oligonucleotide complementary to a target / target nucleic acid., biotin / avidin receptor / ligand and the like.

[048] A "carrier" is a soluble or insoluble polymeric species that can be associated with multiple molecular species at multiple sites through covalent or non-covalent bonds, and/or by encapsulation within a matrix. Example natural or synthetic

polymers that can act as carriers include, but are not limited to, crystals, beads, aggregates, microspheres, oligomers (such as peptides), linear or cross-linked polymers (such as poly lysine, poly acrylic acid, proteins) or highly branched macromolecules (such as dendrimers).

[049] A "cleavable linker" or "cleavable linkage" is a releasable linker that connects two species together, and which contains a linkage that can be specifically cleaved by the action of an enzyme, a particular chemical species, by light or other physiochemical process that cleaves the linker.

[050] A "dissociable linker" or "dissociable linkage" is a releasable linker having at least two domains that can bind to different species where at least one of the domains can be released from one member of its corresponding species and can be re-bound to another member of the same species. A typical example of a dissociable linker is a single stranded nucleic acid having both a poly A tail and a target specific sequence. The poly A tail can be hybridized to a first poly T nucleic acid at one end and to the target nucleic acid at the other end. When the hybrid is melted by temperature or pH, the oligonucleotide can be released from the hybrid and bind another poly T sequence or another target sequence.

[051] A "microsphere" is a particle having a largest dimension of 100 microns or less.

[052] A "particle" is species of a carrier that is insoluble in an aqueous based solvent.

[053] The term "releasable" as used herein with respect to signaling molecules, linkers, adaptors that are associated with a carrier, means that a molecular species can be liberated from association with the carrier by treating the carrier to a condition particularly for the purpose of releasing the molecular species.

[054] A "releasing condition" is to expose the carrier to a physical process or to chemical reagents that will release a particular molecular species from association with the carrier. By way of example, and not by limitation, if the molecular species is non-covalently associated with the carrier by being encapsulated therein, a releasing condition can be dissolving, swelling or crushing the carrier. If the molecular species is associated with the carrier by hybridization between complementary nucleic acids, a releasing condition can be heating the carrier and/or

changing the pH to melt the hybrid. If the molecular species is covalently associated with the carrier through a covalent bond, a releasing condition can be treating the carrier to a chemical or physical process designed to cleave the covalent bond, for example, by using light to cleave a photolabile bond, using an enzyme to cleave an enzymatically labile bond, or using a reducing reagent to cleave a disulfide bond.

[055] A "signaling moiety" is a molecule, or derivative of a molecule, that contains a species having a specifically detectable physical property. In addition, a signaling moiety includes a species that is capable of producing a detectable physical property by interacting with another species. By way of example and not by limitation, typical signaling moieties include chemiluminescent, electrochemiluminescent, fluorescent, chromogenic, and radioactive species. In certain embodiments, an enzyme is also a signaling moiety if the enzyme is capable of reacting with a substrate to generate a detectable chemiluminescent, electrochemiluminescent, fluorescent, or chromogenic product. Thus for example a luciferase or peroxidase may be considered a signaling moiety because when placed in sample containing the appropriate substrates, chemiluminescent products will be produced by the enzyme.

[056] A "substrate" is a solid phase material, which can be attached to a molecular species. A particle is one form of a substrate, as are sample wells, test tube walls, microtiter dishes and the like.

[057] This methods and compositions provided herein relates to a method for improving the sensitivity of analyte detection using a signal amplifying system (SAS). This SAS contains three major components, (a) multiple numbers of signaling moieties (b) a carrier entity or containing the signaling moieties, and (c) one or more analyte binding moieties specific for an analyte. The signalling moieties typically can be chemiluminescent compounds, electrochemiluminescent compounds, fluorescent compounds, chromogenic compounds, radioactive compounds or enzymes that are capable of generating such compounds as a product of an enzymatic reaction with a suitable substrate.

[058] Certain aspects of the methods and compositions provided herein may better understood by referring to Figure 1. In general, the SAS comprises a large number of signaling moieties such as chemiluminescent compounds or their derivatives (such as their precursors) 1, which are attached to a carrier entity 2, which also

carries one or more analyte binding moieties 3 specific for an analyte or analytes. One SAS unit can have multiple copies of chemiluminescent molecules and one or more copies of analyte binding moieties. Preferably the number of the signaling moieties in each SAS unit should be the same or similar for sensitive and reproducible detection. In order to specifically detect analytes, the analyte binding moieties in the SAS need to have specific affinity for the analyte, or for an adaptor that permits specific binding to the analyte or analytes.

[059] Figure 2 depicts one specific embodiment, where the analyte binding moiety 3 is attached to the signaling moiety 1 or their derivatives, which are coupled to the carrier 2.

[060] Conversely, as depicted in Figure 3, the signaling moiety 1 or their derivatives are attached to carrier 2 through the analyte binding moiety 3. The key aspect of these embodiments is that either the analyte binding moiety 3 or the signaling moiety 1, but not both, are directly linked to the carrier.

[061] Figure 4 illustrates a preferred embodiment of the methods and compositions provided herein, where the signaling moiety 1 or their derivatives are encapsulated in microparticle based carrier 2 and the analyte binding moiety 3 are conjugated on the surface of the microparticles 2. This method allows encapsulation of large number of signaling moieties 1 while large numbers of analyte binding moieties 3 can still be labeled to the particle surface.

[062] Figure 5 depicts another preferred embodiment of the methods and compositions provided herein, where the analyte binding moiety 3 or signaling moiety 1 or both are coupled to an intermediate carrier 4 (the second carrier), which is subsequently coupled to the first carrier 2. This method allows provides more signaling moieties 1 per intermediate carrier. This scheme of coupling can be repeated a number of times to provide even more signaling moieties 1. When more than one type of carrier is used, it is preferred that the analyte binding moieties are only coupled to the outer most layer of carrier. It is understood that carriers may be the same type or different type in composition. For example, both first carrier 2 and second carrier 4 can be microparticles. Alternatively, the first carrier 2 may be microparticles whereas the second carrier 4 may be a polymer, e.g., polylysine.

When microparticles are used as carrier 2 and carrier 4, the signaling moiety can be encapsulated into either carrier 2 or carrier 4 or both.

[063] Detection of an analyte is normally accomplished via specific binding of SAS units to a solid substrate such as microwell plate or magnetic particles. After washing away unbound SAS, the bound SAS can be measured immediately for the presence of signaling moieties 1 or after the signaling moieties 1 are released from the carriers. If a chemiluminescent compound is used as the signaling moieties 1, it can be detected using a luminometer or electro-luminometer. The magnitude of amplification is related to the number of signaling moieties on an SAS unit. The more signaling moieties on a carrier entity, the higher the amplification magnitude it can have.

[064] One type of signal moiety is a chemiluminescent moiety. Suitable chemiluminescence here includes, but is not limited to, both direct chemiluminescence such as that generated with enzymes or acridinium and electro-chemiluminescence such as that generated with rare earth elements. Chemiluminescent compounds can be anything that generates a light signal under appropriate conditions or the precursors that give rise to such compounds. Examples for these types of compounds include both chemiluminescent compound (e.g., acridinium and its derivatives, proteins that can generate light, enzymes that can catalyze chemiluminescence reaction) and electrochemiluminescent agents (e.g., certain organic compounds or metal elements in appropriate chelators). It also includes those compounds that function in a fashion similar to a chemiluminescent compound. For example, rare earth elements such as europium emit long half-life fluorescence, which permits time-delayed measurement of the fluorescence after withdrawal of excitation light. Because the fluorescence is measured in the absence of excitation light, these types of compounds function in a fashion similar to a chemiluminescent compound.

[065] Other suitable signaling moieties include, but are not limited to, fluorescent compounds (such as fluorescein), quantum dots, or rare earth elements (e.g., Europium in the form of salt, chelate, oxide, metal etc.), electro-chemiluminescent compounds (such as rare earth elements) and dyes. It is understood that signaling moieties also include the precursors, derivatives, activators or inhibitors of signal

molecules. It is also understood that suitable signaling moieties include those described in scientific journals and other source of public information. .

[066] Signaling moieties 1 such as chemiluminescent compounds may be coupled to the carrier 2, or other components in the SAS, either permanently (non-releasable) or through a cleavable (releasable) linkage, e.g., photo-labile bond, chemical–labile bond such as an acid sensitive bond or a dissociable bond, e.g., polynucleotide base pairing. The cleavable linkage includes, but is not limited to, photo labile bond, chemical sensitive bond, pH sensitive bond, and heat sensitive bond. The signaling moieties can therefore be released using a variety of methods such as oxidation, reduction, acid-labile, base labile, enzymatic, electrochemical, heat and photo labile methods, dissolution and etc. Cleavable linkage may also include non-covalent bonds, which include, but are not limited to, hydrogen bonds (e.g., those in nucleic acid base pairing), ion paring, biotin-streptavidin interaction, and chelating. Under normal assay or storage conditions, the linkage between the signaling moieties 1 and carriers 2, or other component in the SAS to which the signaling moiety is bound, is stable, which permits normal assay procedures such as washing. After unbound signaling moieties are removed, bound signaling moieties 1 are cleaved or otherwise released from the SAS units with desired means. For example, one can use a UV light to cleave UV light sensitive photo labile bond that joints the signaling moieties 1 and the carriers, or other component in the SAS to which the signaling moiety is bound, thereby freeing the signaling moieties to the medium. Detection is then carried out. Release of signaling moieties from the carrier before detection can improve detection efficiency.

[067] An analyte binding moiety can be any chemical or biological functionality with specific affinity for an analyte. They include, but are not limited to, polynucleotides, antibody, antigen, nucleic acid binding species (such as aptamers, which is nucleic acid sequence that can bind with non nucleic acid target), chelators and the like. The analyte binding moiety may be indirectly coupled to the carrier through a linker or an adaptor through, for example, a ligand-receptor binding through binding partners (e.g., biotin-avidin) or hybridization between a polynucleotide and its complementary sequence.

[068] The carrier can be a polymer, a microparticle, or a combination of the two. Appropriate natural or synthetic polymers include, but are not limited to, oligomers (such as peptides), linear or cross-linked polymers (such as poly lysine, poly acrylic acid, proteins) or highly branched macromolecules (such as dendrimers). A chemical, biological or physical entity can be used as a carrier as long as it has multiple functional groups that allow direct or indirect conjugation of multiple numbers of signaling moieties and analyte binding moieties. The more functional groups a carrier has, the better amplification it will provide. An example of the carrier is a microparticle, which can be coated with a large number of functional groups such as carboxyl group or primary amine. Suitable size range of the microparticles includes, but is not limited to, 10 nanometers to 10 micrometers in diameter. Suitable microparticles include, but are not limited to, microspheres, nanoparticles, liposomes, microcapsules and the like.

[069] A carrier may be first directly or indirectly coupled with chemiluminescent compounds or analyte binding moieties or both and then directly or indirectly conjugated to yet another carrier, the second carrier, of the same type or different type. For example, acridinium and an oligonucleotide probe can be first coupled to polylysine to generate an acridinium-polylysine-oligonucleotide probe complex, which is subsequently conjugated to a microparticle. This carrier-to-carrier coupling reaction can be repeated a number of times to achieve further amplification prior to, during, or after contacting the analyte.

[070] When microparticles or the like are used as carriers, signaling moieties can be encapsulated in the particles. Encapsulation may be performed through physical means, e.g., trapping, internal adsorption, or through chemical means, e.g., covalent coupling. Alternatively, signaling moieties can first be directly or indirectly coupled to a carrier (e.g., a polymer or nanoparticles) and then encapsulated in the particle. When signaling moieties are encapsulated in particles, the particles can be dissolved, swelled, or perforated to release the signaling moieties or make them more accessible to trigger reagents. These treatments can improve the sensitivity. One could use certain physical means or certain chemicals (such as organic solvent, strong acid or base, or heat) to swell or partially or completely dissolve or destroy the microparticles to release the trapped signaling

moieties. For example, polymer microspheres made from monomers containing high concentration of 4-amino styrene or acrylic acid can be dissolved with acid or base respectively, similar to the method used for controlled release in pharmaceuticals. The encapsulated signaling moieties could be in the form of aggregate, e.g., small particles, powder, or crystals, which are preferably in nano meter size range. For example, when rare-earth element such as Eu is used, it could be in the form of Eu metal particles, Eu oxide particles or other Eu containing compounds aggregate. The SAS particles containing these forms of Eu or other rare earth elements are also useful for fluorescent detection or electro-chemiluminescent detection. The encapsulated rare elements can be released from the particles using physical means or certain chemicals (e.g., organic solvent, strong acid or base). Suitable chemicals for encapsulating chemiluminescent or fluorescent compounds include, but are not limited to, polymers such as polystyrene. In certain embodiments, small organic compounds such as Si-containing compounds can be used to coat chemiluminescent or fluorescent compound particle to give the encapsulated SAS microparticles. For example, 3-aminopropyltrimethoxysilane APTMS can be used to coat Eu_2O_3 nano particle or the like. The coated Eu_2O_3 particles or the like can be conjugated with analyte binding moieties and used as an SAS in an assay. In some embodiments, the particles are treated with certain chemicals (e.g., acid, organic solvent in acidic condition) to dissolve the particles and release Eu into a soluble form prior to fluorescence detection; a fluorescence-enhancing agent such as beta-diketone is preferably, but not necessarily, used for the detection of Eu fluorescence. In yet other embodiments, the released Eu or the like is used for electro-chemiluminescent detection.

[071] Rare earth elements or the like may be encapsulated in microparticles in the form of ions through a chelator. The Eu chelate, e.g. EDTA-Eu, beta-diketone-Eu, TTA-Eu-TOPO can be incorporated into the monomer covalently. Appropriate chelators include, but are not limited to, isothiocyanatobenzyl-EDTA, TTA derivatives with an acid group, EDTA-5-aminosalycilic acid, and 4,7-bis (chlorosulfophenyl)-1, 10-phenanthroline-2,9-dicarboxylic acid (BCPDA). These chelators can be coupled to amine containing monomers such as 4-amino styrene through a covalent bond (e.g., amide bond) linkage. The resultant monomer

containing an Eu chelate can be subsequently polymerized into microparticle or copolymerized with other monomer to give desired microparticles. Alternatively, the chelator, e.g., EDTA, diketone, can be first covalently incorporated into the monomer, which is then polymerized or copolymerized with other monomer to form desired microparticles. The microparticles are then swelled or perforated and then incubated with Eu salt solution to allow Eu incorporation via the chelator molecules. These microparticles containing the diketone-Eu chelate allows high sensitive fluorescent detection even without releasing the Eu from the particles.

[072] The more signaling moieties are there in a SAS microparticle, the better detection sensitivity can be achieved. Microparticles with larger sizes can normally carry more signaling moieties and therefore will result in better detection sensitivity. Preferably, the microparticles (such as polystyrene microspheres) in an SAS are larger than 10 nanometers in diameter. More preferably, the microparticles are larger than 100 nanometers in diameter. In our study, the microparticles as large as 1~3 micrometers in diameter can give satisfactory detection sensitivity. It is preferable that millions or more of signaling moieties to be coupled or entrapped to these microparticle for very high amplification effect.

[073] The microparticles can be directionally labeled with the analyte binding moieties and signaling moieties so that the analyte binding moieties are present in the outermost layer while the signaling moieties are in a layer beneath the analyte binding moiety layer. An example is illustrated in Fig. 6; in which signaling moieties 1 and analyte binding moieties 3 are directionally labeled on the microsphere 2 via polymer backbone 5. This layered or directional labeling method minimizes physical hindrance of the signaling moieties 1 with analyte binding thereby improving analyte binding efficiency.

[074] For directional labeling one can use a polymer or polymers as the carrier to which both signaling moieties 1 and analyte binding moieties 2 can be labeled. Linear polymers (e.g., poly lysine, poly acrylic acid or modified nucleic acids) or highly branched macromolecules (such as dendrimers) can be used as the polymer backbone. They could be either natural or synthetic. One end of the polymer is attached to the microsphere whereas another end is linked to one or more analyte binding moieties. The signaling moieties 1 such as the fluorescent or

chemiluminescent groups are normally conjugated to the polymer backbone or side chain in between the two termini. The linkage between the signaling moieties and the polymer can also contain one or more cleavable bonds that can be cleaved using certain chemical (e.g., a strong acid) or physical means (e.g., UV irradiation).

[075] Typically, but not necessarily, the linkage between the polymers and particles and between polymer and analyte binding or signaling moieties involves covalent bonds. In certain situations, noncovalent linkages may provide a convenient alternative. For example, analyte binding moiety may be linked to the polymer via biotin-avidin binding, as illustrated in Figure 7. In this case, the microparticles 2, which are labeled with biotin or avidin, are not analyte specific. An analyte binding moiety such as antibody AB is labeled with avidin or biotin and then bound to the microparticles labeled with biotin or avidin via polymer 5, respectively. In order to ensure that the analyte binding moieties are located on the outermost surface of the microparticles, which provides faster and more efficient binding to the analyte, a hydrophilic group such as a Poly Ethylene Glycol fragment can be attached to a position, or positions, at or near the outer terminus of the polymer. Fig 7 illustrates a versatile frame for this layered signal amplifying microparticle, where the analyte binding moiety (e.g., antibody AB) is attached to the polymer terminus through an interaction between corresponding binding partners (e.g., biotin : avidin), one of which is coupled to the analyte binding moiety. It is understood that the ligand-receptor interaction can be replaced with other types of binding such as nucleic acid hybridization.

[076] When used for analyte detection, the SAS can be used in a "sandwich" format or its variations. Generally, an analyte is first immobilized onto a solid phase using an analyte binding moiety such as an antibody, which preferably binds to a different epitope than the analyte binding moiety on the SAS. After wash away unbound entities, the SAS is added to a binding solution that permits the binding of SAS to immobilized analytes. After washing away unbound SAS, the bound SAS is detected by its associated signaling moieties using an appropriate instrument such as a luminometer. In certain embodiments, the mixing and binding of analyte to solid phase and SAS are performed in one step simultaneously.

[077] In some embodiments, the SAS or the signaling moieties is separated from the solid phase capture surface (e.g., micro plate well or magnetic beads) before detection, which may reduce potential interference from the capture surface since the capture surface itself can produce significant background (e.g., background fluorescence) or the particles can block a light signal. Physical means (e.g., heat) or chemical means (e.g., appropriate acid or base, protein denaturing reagents, e.g., guanidine isothiocyanate, or the like could be used to disassociate the sandwich structure or to release the signaling moieties from the SAS, thereby separating the signaling moieties from the capture surface. If the SAS microparticle dissolution step is involved during the assay, the magnetic capture particles, if used, can be made resistant to the dissolution condition by using, for example, magnetic beads made of highly cross-linked polymer, which allows the separation of magnetic particles from SAS or signaling moieties. In microwell plate based assays, the capture surface is normally coated with analyte binding moieties, e.g., antigens or oligonucleotides, which often generate background fluorescence. In this situation, the dissociated SAS or signaling moieties or fluorescent probes are preferably transferred to a clean well for detection.

[078] If the signaling moiety is an organic chemiluminescent compound, a luminometer can be used for detection; if the signaling moiety is electro-chemiluminescent agent such as rare earth element, an electro-chemiluminescent detector can be used for detection. If the signaling moieties are coated on the surface of the particles, it is not necessary, though preferred, to release them to the solution prior to detection.

[079] In certain embodiments, the SAS is used in combination with other instruments. For example, it could be used in combination with a wave-guide sensor like device to further increase its detection limit.

[080] The above methods use SAS only once in the detection, it is essentially a single round amplification method. In certain modifications to the above SAS and detection protocol, multiple rounds of amplification can be achieved by using two or more SAS sequentially. In a multiple rounds of amplification, there are several different types of SAS. Among these different types of SAS, one of the SAS types normally, but not always, contains the signaling moieties whereas the

other SAS types contain markers for SAS detection (i.e., SAS detection markers) instead of the signaling moieties.

[081] The concept of multiple rounds of SAS amplification can be better understood by referring to Figure 10, which illustrates an amplification process that involves two types of SAS, the first SAS 21 and second SAS 24. The first SAS 21 is coated with an analyte binding moiety 22 and a binding partner 23. The second SAS 24 is encapsulated with signaling moieties and coated with another binding partner 61 that is specific for the binding partner 23 on the first SAS 21. Here in this particular example, the capture surface is a magnetic particle 25 is coated with another analyte binding moiety 60, which may or may not be the same as analyte binding moiety 22. In the presence of an analyte, the magnetic particle 25 binds to the first SAS 21. After washing to remove unbound first SAS 21, the second SAS 24 is added to the solution, which binds to the first SAS 21 through the binding partners on the first SAS 21. After washing to remove unbound second SAS 24, the signaling moieties encapsulated in the two SAS are released for detection. Therefore, one analyte could result in the binding of a large number of second SAS 24.

[082] Alternatively, the binding partner can be replaced with a releasable linker or adaptor. The releasable linker also contains a binding partner for the SAS, which could be for example, single or double strand DNA, RNA, PNA (peptide nucleic acid) or the like, antibodies, antigens, small molecules, the analyte binding moieties themselves or any chemical entities as long as they can be bind to another SAS system used in the next round of amplification. The releasable linker could be either encapsulated inside or bound on the surface if microparticles are used as carriers. In certain embodiments, the releasable linker in the first SAS is the analyte binding moiety itself, in which case there is no need to have distinct releasable linker. Specific examples are given the Example section below.

[083] In certain embodiments, the releasable linker in the first SAS is the analyte binding moiety itself, e.g., an antibody. In these embodiments, the second round of amplification involves the second SAS, which are coated with multiple aptamers that have affinity for the antibody resulted from the first SAS. The second SAS is added to the resultant reaction mix of the first amplification round. After

binding and washing to remove unbound second SAS, the aptamers are released from the second SAS to achieve a second round of amplification. Similarly, the third round amplification uses the third SAS having multiple DNA sequence complementary to the released aptamers. Again after binding and washing, huge amount of DNA copies can be released from the third bounded SAS. If the amplification is great enough, the resulting DNA can be detected using traditional detection methods or another SAS system having chemiluminescent compounds or fluorescent compounds. Alternatively more rounds of amplification can be achieved using more of this kind of SAS before the final detection. In this example, the releasable linker is the binding moiety (e.g., analyte binding moiety). It can also be other molecules, for example, one SAS having antibody as the analyte binding moiety and DNA or PNA as the releasable linker. The higher the amount of SAS releasable linkers per SAS unit, the higher the amplification power one can achieve in each round. Preferably, one unit of SAS can carry more than one thousand copies of the releasable linker.

[084] EXAMPLE 8 (Figure 12) further explains these embodiments, in which the releasable linker in the first SAS is the analyte binding moiety itself.

EXAMPLE 1

Signal amplification with release of signalling moiety

[085] FIG. 8 illustrates one example in which SAS technology is used for analyte detection. The assay is aimed to detect a certain antigen 11 in the sample containing other molecules 10, *i.e.*, the non-target molecules. The micro well plate well surface 7 is coated with antibody 6 specific for the antigen 11 using a method known in art. The microsphere-based SAS 9 contains another antibody 12 specific for an epitope distinct from that for antibody 6 on the microwell plate wells. In addition, SAS 9 also contains chemiluminescent molecules 8 as the signaling moieties. In the presence of antigen 11 in the sample and under appropriate binding conditions (e.g., appropriate buffer, temperature etc.), some of the SAS 9 are immobilized on the surface of the microwell plate well 7 through a sandwich binding in which the antigen interacts with both the SAS 9 and microwell plate. After washing to remove the unbound SAS 9 and analyte 11, the chemiluminescent

molecules 8 are released from the SAS 9 using chemicals or light that can cleave the bonds between chemiluminescent molecules 8 and the microsphere. The released chemiluminescent molecules 8 can be readily detected using a luminometer or electro-chemiluminescence detector. Although releasing is preferred, the bound chemiluminescent compounds can also be detected without being cleaved. The chemiluminescence intensity is proportional to the amount of antigen 11 in the sample.

[086] The chemiluminescent molecules 8 can also be replaced with fluorescent molecules as signaling moieties. The fluorescent molecules may be coupled to the particles through a linker containing a disulfide bond (e.g., dithiopropionate), which is thiol-cleavable. Upon binding, the unbound microspheres are removed from the well through washing. Then the fluorescent molecules are cleaved from the microspheres using a reducing agent such as beta-mercaptoethanol. The released fluorescent molecules can be readily detected using a fluorometer. The fluorescence intensity is proportional to amounts of bound microspheres, which are in turn proportional to the amount of antigen in the sample.

[087] In certain embodiments the signaling moieties are coupled to the inside of the microparticle via cleavable linker if the microparticle is porous or solvent permeable. In other embodiments, the signaling moieties such as the chemiluminescent agents or fluorescent agents can be coupled to the inside of microparticle via non-cleavable linker since they can still give detectable signal (such as light) without being released.

EXAMPLE 2

Magnetic particle based signal amplification for detecting HIV RNA

[088] Figure 9 illustrates yet another example for using SAS in an assay. In this case, magnetic particles 18 are used as the solid phase substrate. The chemiluminescent molecules 15 are encapsulated in the microparticles 16. Magnetic particles 18 and SAS 17 are coated with distinct analyte binding moieties, e.g., polynucleotide probes 13 and 14 that hybridize with different regions of HIV-1 viral RNA 20 for the detection of this virus. The magnetic particles are preferably approximately 3 micrometer in diameter and are coated with functional groups such

as carboxyl group, which facilitates the labeling of analyte binding moieties such as oligonucleotide probe 13. An example for suitable magnetic particles is Dynabeads M-270 coated with carboxylic acid (available from Dynal Biotech, Oslo, Norway). Dynal Biotech provides a protocol for labeling of oligonucleotides to the magnetic particles.

[089] An assay requires at least one set of probes, e.g., polynucleotide probes 13 and 14, although more than one set of probes is preferred since more probes may provide stronger binding. The probes may not necessarily need to be conjugated to magnetic particles or SAS unit if the probes also contain a suitable binding partner, e.g., biotin, or a specific nucleic acid sequence, which can be used for binding to magnetic particles or SAS units through ligand-receptor binding or nucleic acid hybridization.

[090] The sample to be tested is first treated with appropriate reagents and conditions, e.g., a buffer containing guanidine thiocyanate, to denature the proteins and release the nucleic acids in the sample. Magnetic particles 18 are added and incubated for an appropriate period time (e.g., 60 minutes) at appropriate temperature (e.g., 50 degree C) in an appropriate buffer (e.g., 0.1 M PBS buffer) that promotes nucleotide hybridization. The capture magnetic particles 18 are then washed several times to remove unbound entities 19, suspended in an appropriate buffer that promotes specific hybridization, and then incubated with SAS microparticles 17. If HIV-1 RNA 20, or an appropriate portion of it, is present, the magnetic particles 18 and SAS microparticles 17 will be bound together through HIV-1 RNA 20. After washing away unbound SAS microparticles using a magnet or its equivalent, the bound SAS microparticles are dissolved with an appropriate solvent, e.g., dimethylsulfoxide (DMSO) for the polystyrene particles, and released chemiluminescent compounds 15 are detected with an instrument such as luminometer.

[091] Because only a few analyte molecules are needed to provide stable binding between magnetic particles 18 and SAS particles 17 and because each SAS microparticle 17 is encapsulated with a large number of chemiluminescent molecules 15, the signal is greatly amplified. The sensitivity of the assay depends on several factors, including the minimal number of SAS microparticles 17 that can

be detected and the efficiency of removing unbound SAS microparticles 17 (the background). For example, if the minimal number of SAS microparticles 17 that can be detected is ten (10) and all unbound SAS particles 17 are removed, then the sensitivity of the assay is ten HIV-1 RNA copies. When the magnetic particles 18 and polystyrene microparticle based SAS 17 labeled with acridinium as chemiluminescent molecule are used for detecting HIV-1 viral RNA 20, as low as ten copies of virus can be detected. To increase the stability of the magnetic particles 18-HIV-1 RNA 20-SAS 17 complex, multiple pairs of probes are used with each pair hybridizing to different regions of the HIV-1 RNA 20. Preferably, but not necessarily, one probe in a pair is coupled to magnetic particle 18 whereas the other probe in a pair is hybridized to the SAS 17. The use of multiple different probes on each magnetic particles 18 and SAS unit 17 can improve the sensitivity for HIV-1 RNA detection. In this case even one copy of HIV-1 RNA 20 could result in multiple polynucleotide binding pairs between the HIV-1 RNA 20 and magnetic particles 18 and between the HIV-1 RNA 20 and the SAS 17.

EXAMPLE 3

Detection of bacteria

[092] This example shows how the SAS technology can be used for sensitive detection of a particular species or class of species of bacteria using a nucleic acid target, e.g., tRNA, ribosomal RNA. Similar to the HIV-1 assay, there needs to be at least one pair of probes. Here in this example, the probes are relatively long oligonucleotides that contain two hybridization domains, one of which is specific for the target nucleic acids whereas the other domain is specific for the oligonucleotides conjugated on magnetic particles for one of the probes or for the oligonucleotides conjugated on SAS units for another probe. In this example, we use Probes A and B, which contain hybridization domains for magnetic particles and SAS, respectively. The magnetic particles are preferably approximately 3 micrometer in diameter and are coated with functional groups such as carboxyl group, which facilitates the labeling of analyte binding moieties such as oligonucleotide probe. An example for suitable magnetic particles is Dynabeads M-270 coated with carboxylic acid (available from Dynal Biotech, Oslo, Norway).

[093] Teachings for preparing various components for the assay were described in U.S. provisional patent application 60/555,683, and U.S. patent application No. 10/205,195, each incorporated here by reference.

[094] The sample to be tested is mixed with 2 volumes of appropriate lysis buffer, e.g., 2 mL 50 mM Tris-HCl, pH 7.4, 5 M guanidine thiocyanate and 2% Triton X-100, and rotated at room temperature for 20 minutes. Appropriate amounts of at least one pair of probes, e.g. 10^9 copies in 1 mL PBS buffer, which hybridize to different regions of the target RNA, are added to the lysed sample. The reaction mix is heated to 94 degree for 5 minutes and then incubated at 50 degree for 15 to 60 minutes to allow the probes to anneal to the target nucleic acids, which results in the formation of Probe A-Target RNA-Probe B complex. After addition of an appropriate amount of magnetic particles, e.g., 10^7 particles, the reaction mix is incubated for 15 to 60 minutes with agitation to allow the hybridization of all Probe A, which contains the hybridization domain for the polynucleotide probe on the magnetic particles. The reaction solution is then removed using a magnet. The magnetic particles are washed three times with 1 to 2 mL of washing buffer, e.g., phosphate saline buffer (PBS). If there is a sufficient amount of target nucleic acids in the sample, the magnetic particles will be labeled with Probe B through its binding to target nucleic acids.

[095] To detect Probe B bound to the magnetic particles, the washed magnetic particles are suspended in 100 microliters hybridization buffer, e.g., PBS with 10 mM aminoethanethiol, 2% Tween 20. After addition of appropriate amounts of SAS, e.g., 10^6 particles, which is conjugated with an oligonucleotide that can hybridize with Probe B, the mix is incubated for 15 to 60 minutes under appropriate conditions that promote specific hybridization. The magnetic particles are then washed to remove unbound SAS. The bound SAS is detected through an appropriate instrument such as a luminometer. Alternatively, the bound SAS can be released from magnetic particle by mixing with 1 mL 0.1 N HCL for 3 minutes before detection, which can enhance the efficiency of signal detection.

EXAMPLE 4

Encapsulation of acridinium in a microparticle

[096] This example teaches a method for encapsulating acridinium, or its derivatives, into microparticles that can be used as part of the SAS. Because of the charge present in a typical acridinium molecule, a sufficiently hydrophobic moiety or moieties are preferably attached to acridinium, thereby creating an acridinium derivative that is sufficiently hydrophobic, *i.e.*, substantially insoluble in aqueous solution. Such a hydrophobic acridinium species minimizes the leaching from inside the microparticles under aqueous conditions once it is encapsulated inside the microparticles.

[097] Hydrophobic acridinium derivatives are normally dissolved in an organic solvent so that a concentrated acridinium solution can be created for encapsulation. The microparticles used for encapsulation should be compatible with the organic solvent, *i.e.*, the basic structure of microparticles should not be partially or completely dissolved, or otherwise significantly altered. For example, polystyrene-based microparticles may be dissolved in certain organic solvents such as chloroform, but would be compatible with the others such as ethanol. Polystyrene microparticles copolymerized with a cross-linking polymer(s) may be compatible with most organic solvents, including dichloromethane and chloroform. The microparticles are preferably, but not necessarily, functionalized with functional groups such as primary amine or carboxyl group. The following procedure teaches one method for encapsulating acridinium or its derivatives in microparticles. It is understood that different methods, or variations of the current method, can also be used to achieve sufficient encapsulation of acridinium or its derivatives.

[098] This procedure teaches the encapsulation of an acridinium derivative, 4-dodecylphenyl–10-methylacridinum-9-carboxylate trifluoromethane sulfonate, in a cross-linked polystyrene microparticles (Spherotech, Inc, catalog number APX-20-10, 2.48 micrometer in diameter), which is functionalized with primary amines. Prior to encapsulation, the microparticles are washed twice with dry ethanol (100%) and then twice with acetone. One hundred milligrams of the acridinium derivative is dissolved in 1. 0 mL CH_2Cl_2 (dichloromethane) to make 10% (100 mg/mL) solution, which is used to suspend 50 mg cross linked polystyrene particles prepared

as described above. The microparticle solution is then rotated in a rotating device for 30 minutes at room temperature. The microparticles are then filtrated through a 0.1 micrometer VVPP filter (Millipore catalog number VVLP04700) and washed with 10 mL of 50% ethanol aqueous solution three times. The microparticles are then dialyzed in 1 L PBS buffer overnight to remove unencapsulated acridinium. The dialysis step is repeated once or more. The resulting microparticles are now encapsulated with the acridinium derivative. The functional groups on the microparticles surface can be used to directly or indirectly couple with analyte binding moieties. Alternatively, a binding partner linked to an adaptor can be directly or indirectly conjugated to the microparticles. The resultant SAS can be used for analyte detection. It is preferred, but not necessary, to release the encapsulated acridinium derivative prior to chemiluminescent detection during an assay by incubating for two minutes with an organic solvent such as DMSO.

EXAMPLE 5

Encapsulation of Eu in a microparticle

[099] This example uses a rare earth element, Eu-beta-diketone-TOPO chelated complex that is encapsulated in microparticles or microspheres, as SAS for electrochemoluminecescent-based analyte detection. The surface of the microparticles or microsphere is coated with analyte binding moieties. Prior to electrochemoluminescence detection step after the assay (e.g., the assay described in example 3), the encapsulated Eu is released from the particles by mixing with organic solvent (e.g. 1 mL acetone) for 2 minutes to improve detection efficiency. The released Eu can also be used for fluorescent detection.

[0100] Alternatively, the oxides of rare elements, e.g., Eu oxide microparticles, can also be directly coated with amine group using known 3-amino-propyltrimethoxysilane chemistry. The resulting amine groups are used for affinity group labeling. These Eu oxide microparticles are used as SAS for analyte detection. In the detection step of the assay, the resulting Eu oxide microparticles are dissolved with 1N HCL aqueous solution for measuring its electrochemoluminecescent activities.

EXAMPLE 6

Multiple rounds of amplification

[0101] This example teaches a method for multiple rounds of amplification processes for achieving event greater signal amplification. This example is better understood by referring to Figure 10, which depicts a two-round amplification process using two SAS types and one magnetic capture bead type.

[0102] As shown in Figure 10, both SAS 21 and SAS 24 are encapsulated with chemoluminescent compounds. The first SAS 21 is coated with an analyte binding moiety 22 and a binding partner 23, e.g., biotin. The second (and last) SAS 24 is coated with only one binding partner 61, which is specific for binding partner 23 on the first SAS 21, e.g., avidin or its derivatives.

[0103] To conduct an assay, the analyte to be detected is first immobilized onto a solid phase such as magnetic particles 25 shown in Figure 10. The magnetic particles 25 are then incubated with first SAS 21 under conditions that promote specific binding between SAS 21 and magnetic particles 25 via the analyte. After removal of unbound SAS 21 through washing, the second SAS 24 is added and incubated again to allow the binding of SAS 24 with SAS 21 through the binding partners, e.g., biotin-avidin binding. After removal of unbound SAS 24, the encapsulated chemoluminescent compounds in both SAS 21 and SAS 24 are released for detection.

[0104] It is evident that more than two rounds of amplification can be accomplished by simply designing additional SAS with appropriate binding partners, which can be DNA (deoxynucleic acids), RNA (ribonucleic acids), PNA (peptide nucleic acids), or other ligand-receptor binding system.

EXAMPLE 7

Amplification with releasable linkers

[0105] This example (Fig. 11) illustrates yet another example of multiple rounds of amplification processes. When compared to Example 6, the difference in current example is in the composition of the first SAS 26, which is coated or encapsulated with a larger number of a releasable linker that has two binding domains. The releasable linker can be DNA, RNA, PNA, ligand-receptor binding system, an antigen with at least two epitopes, or a combination of the two. The more

releasable linkers are there on SAS 26, the higher amplification power will it have. Preferably, more than 1,000 releasable linkers are coated on or encapsulated into each SAS 26 unit.

[0106] The first SAS 26 is coated with a releasable linker 29 in addition to an analyte binding moiety 50. The releasable linker 29 is an oligonucleotide (oligo dA) coupled with biotin and therefore has two binding domains: oligo dA and biotin. The oligo dA is preferably, but not necessarily, 20 base or longer. The releasable linker 29 is attached to SAS 26 through complementary hybridization with oligo dT, which is covalently conjugated to SAS 26. The second SAS 27 is similar to that in EXAMPLE 5 in that it is coated with a binding partner 51, e.g., avidin that binds to a domain in releasable linker 29, e.g., biotin. SAS 27 is coated or encapsulated with a signaling moiety, e.g., a chemiluminescent compound.

[0107] To conduct an assay, the analyte to be detected is first immobilized onto a solid phase such as first magnetic particles 28, which is coated with an analyte binding moiety for capturing the analyte. The magnetic particles 28 are then incubated with first SAS 26 under conditions that promote specific binding between SAS 26 and first magnetic particles 28 via the analyte. After removal of unbound SAS 26 through washing, releasable linker 29 is released from SAS 26.

[0108] The method for releasing the releasable linker 29 depends on how it is coupled to SAS 26. Appropriate coupling methods include, but are not limited to, photo-labile bond, reducing agent sensitive bond and nucleic acid hybridization. If the linker is coupled to SAS through a photo-labile bond, then the reaction mix can be subjected to irradiation of appropriate light. If the coupling bond is sensitive to reducing agent as in the case of disulfide bond, then the reaction mix can be subjected to reducing agent. In this example, the linker is coupled to SAS 26 through oligo dA:oligo dT hybridization. Therefore, releasable linker 29 can be released by adjusting the pH or, preferably, temperature. There is now a large number of releasable linkers in the reaction even if there is only a limited number of specifically bound SAS 26.

[0109] The assay then proceeds to second amplification step, which uses releasable linker 29 as an analyte in the reaction solution. A number of methods can be used to detect the released releasable linkers. Described here is one example.

Second magnetic particles 30 are added to the reaction mix preferably, but not necessarily, immediately before the releasable linker 29 release step. Magnetic particles 30 are coated with oligo dT, which is preferably 25 base or longer. The amount of magnetic particles 30 in the reaction should satisfy the desired upper and lower limit of detection, and will likely need to be optimized depending on a particular assay (e.g.; in significant excess of the amounts the number of bound SAS 26 multiplied by the number of the linker on one SAS 26 unit, preferably with a ratio of at least 10 to 1 in unit numbers). The reaction mix is then heated to a temperature at which releasable linker 29 can be released from SAS 26. The temperature is then lowered to a temperature that permits hybridization between oligo dT and oligo dA. Because second magnetic particles 30 greatly outnumber SAS 26, most releasable linker 29 will hybridize with the second magnetic particles 30 even though SAS 26 is also coated with oligo dT. The second magnetic particles 30 are now coated with biotin, which is conjugated to releasable linker 29. It is not necessary, but may be preferred, to wash away SAS 26 before proceeding to the next step.

[0110] The second SAS 27, which is coated with avidin and signaling moiety (e.g., chemiluminescent compounds), is then added to the reaction mix. After an incubation to allow avidin-biotin binding, the unbound SAS 27 is thoroughly washed away. The bound chemiluminescent compounds can be detected with an instrument such as a luminometer. The amounts of analyte in the sample are proportion to the signal within linear range of the assay.

[0111] The procedure described above is essentially a two round amplification assay, the assay condition in each round can be similar to those described in EXAMPLE 1~3. Evidently more than two rounds of amplification can be performed with the use of more linkers. It is within the scope of this methods and compositions provided herein that signaling moieties may be something other than chemiluminescent and electro-chemiluminescent compounds. Appropriate signaling moieties include, but are not limited to, fluorescence generating compounds, color generating compounds, and enzymes that may generate such signals (e.g., peroxidase).

[0112] The amplification power primarily depends on how many linker molecules are incorporated into the first SAS 26 and how many signaling moieties are incorporated into the second SAS 27. For example, if 10,000 linker molecules are incorporated to the first SAS 26 and 10,000 signaling moiety molecules are incorporated into the second SAS 27, then the amplification power would be 100 million with respect to the number of analyte molecules.

EXAMPLE 8

Amplification with a releasable analyte binding moiety

[0113] This example (Fig 12) illustrates a yet another method of multiple rounds of amplification. This method is similar to that disclosed in EXAMPLE 7 except that the releasable linker itself has an analyte binding moiety, which can be a nucleic acids, antigen, antibody, ligand, receptor, aptamers, or any analyte binding moiety that can provide specific binding for an analyte, or a combination of the two. The releasable linker can be released from the first SAS 31 under conditions that preserve the binding capacity of the linker. Figure 12 depicts an assay using this amplification method.

[0114] To conduct an assay for the detection of an analyte, the analytes are first immobilized on a solid phase such as magnetic particles 32, which is coated with an analyte binding moiety. The analyte-magnetic particle complex is then incubated with the first SAS 31, which is coated with the analyte binding moiety 52, which also contains at least two specific binding domains, one for the analyte and another for linker binding moiety 53 on the second magnetic particle 34 or for linker binding moiety 54 on the second SAS 33. The analyte binding moieties on the first SAS 31 and first magnetic particles 32 are specific for different regions of the analyte. Magnetic particles 32 and SAS 31 can be added to the sample simultaneously, not necessarily sequentially.

[0115] After washing away unbound first SAS 31, the analyte binding moiety 52 are dissociated under a condition that preserves their binding capacity for the next round amplification. The second SAS 33 and second magnetic particles 34, which are coated with linker binding moiety 54 and 53, respectively, are added to the solution. Although in some cases the second magnetic particles 34 and first SAS 31 can compete for the same binding site on analyte binding moiety 52 if the same

binding site is used, most linker will bind to the second magnetic particles 34 since the second magnetic particles vastly outnumber the first SAS. The second SAS 33, analyte binding moiety 52 and second magnetic particles 34 form a complex in a sandwich format, which allows the removal of unbound second SAS 33 using, for example, a magnet. The signaling moiety conjugated on or encapsulated in the second SAS 33 is released under appropriate conditions depending on the nature of the carrier and method of signaling moiety conjugation or encapsulation. The signal is detected using appropriate instrument.

[0116] The number of captured second SAS 33 depends on the number of analyte binding moiety in the solution, which in turn depends how many analyte binding moiety are coated onto the first SAS 31 and how abundant the analyte is. Because each first SAS 31 can be coated with numerous analyte binding moiety, the signal is greatly amplified. For example, if 10,000 linker molecules are incorporated to the first SAS 31 and 10,000 signaling molecules are incorporated into the second SAS 33, then the amplification power would be 100 million with respect to the number of analyte molecules.

[0117] From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the following claims.